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## Colchicine-Induced Modulation of Collagenase in Human Skin Fibroblast Cultures. I. Stimulation of Enzyme Synthesis in Normal Cells

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Microtubule-active agents affect the secretion of a variety of proteins, including collagenase. To gain insight into the mechanisms involved in this process, we examined the effects of colchicine on the synthesis, secretion, and activity of human skin collagenase. When added to monolayer cultures of human skin fibroblasts,  $10^{-6}$  M colchicine produced a mean 3-fold increase in trypsin-activatable collagenase in the culture medium. Stimulation was not observed with lumicolchicine. The enhanced accumulation of collagenase was dose-dependent with  $10^{-9}$ ,  $10^{-8}$ ,  $10^{-7}$ , and  $10^{-6}$  M colchicine giving collagenase activities/mg protein that were  $100 \pm 6\%$ ,  $165 \pm 20\%$ ,  $186 \pm 34\%$ , and  $297 \pm 62\%$  of control, respectively. Although the effect on collagenase was seen under conditions independent of cellular growth (i.e., in serum-free medium), maximum stimulation occurred in subconfluent cultures. The colchicine-induced increase in activity was paralleled by an increase in immunoreactive enzyme protein, suggesting stimulation of enzyme synthesis. The catalytic efficiency of the enzyme (activity per unit immunoreactive protein) was unchanged, however, indicating that a structurally normal enzyme was being synthesized. To examine the process in more detail, the biosynthesis of  $^3\text{H}$ -labeled collagenase was quantitated in these cultures by specific immunoprecipitation. Although  $10^{-6}$  M colchicine produced no increase in total protein synthesis, an increased rate of collagenase synthesis was seen after only 1.5 hr. These data suggest that colchicine has a specific effect on the synthesis of colla-

genase and may be a useful probe for studying its regulation.

Human skin fibroblasts synthesize and secrete collagenase as one of their major extracellular gene products [1]. This enzyme, which is the critical enzyme in the initiation of physiologic collagen degradation [2], is also important for its presumed roles in the hereditary blistering disorder, recessive dystrophic epidermolysis bullosa, and in the facilitation of soft tissue destruction in cutaneous neoplasia [3]. In each of these disorders, collagenase has been found to be present both in vivo [4-6] and in vitro [7-12] in increased concentrations. Thus, in order to gain further insight into the cellular mechanisms leading to increased collagenase expression, i.e., enhanced synthesis and/or activity, we have investigated the effects of certain exogenous agents on this biochemical trait.

In this regard, agents which act on microtubules—such as colchicine—are known to affect the synthesis and secretion of various proteins. However, the effects of this drug are in no way uniform or predictable from system to system. For example, in calvarium explant cultures and in fibroblast cultures, colchicine leads to a marked decrease in secretion of procollagen which may be associated with diminished synthesis [13-16]. Similarly, in chondrocyte cultures microtubule-active agents cause decreased secretion of proteoglycans [17]. In contrast, micromolar concentrations of colchicine have been shown to stimulate plasminogen activator in Swiss 3T3 cells [18] and to enhance the release of collagenase in explant cultures of rheumatoid synovium [19] and in cultures of mouse peritoneal macrophages [20]. From these latter two studies it would seem that this drug would be an ideal agent for exploring mechanisms of collagenase stimulation in human skin fibroblasts.

### METHODS

#### *Fibroblast Cultures*

Human skin fibroblast cultures were initiated from a 3-mm punch biopsy of normal volunteers after obtaining informed consent, or were purchased from the American Type Culture Collection (Bethesda, Maryland). Cells were subcultivated at  $37^\circ\text{C}$  in Dulbecco's modified Eagle's medium—high glucose + glutamine. This medium was supple-

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#### Abbreviations:

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

mented with 0.03 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) buffer (pH 7.6), 15% fetal calf serum, and antibiotics (penicillin and streptomycin, 200 U per ml and 200 µg per ml, respectively). Upon attaining the desired state of growth, the cells were washed 3 times with Hanks' balanced salt solution and placed in the same medium without fetal calf serum (serum-free medium) containing varying amounts of colchicine for periods of up to 24 hr as described previously [21,22].

#### Collagenase Activity

Collagenase activity was measured in the fibroblast culture medium as previously described [23]. Briefly, 100-µl portions of medium containing latent collagenase were proteolytically activated for 10 min at 25°C with a range of trypsin concentrations to insure that maximum activity was measured. Tryptic activity was then stopped with a 5-fold excess of soybean trypsin inhibitor, and the entire mixture was assayed at 37°C using native reconstituted guinea pig skin collagen fibrils labeled with [<sup>14</sup>C]-glycine [24].

#### Immunoreactive Collagenase

Immunoreactive collagenase protein was quantitated using the double-antibody radioimmunoassay previously reported [25]. The enzyme used for iodination and for developing the standard curve was human skin procollagenase purified by the method of Stricklin et al [26]. This enzyme preparation was also used to elicit functionally specific antiserum to the enzyme [27].

#### Biosynthetic Studies

The biosynthesis of human skin collagenase was carried out as described [1,10]. Briefly, monolayer cultures were labeled with serum-free medium containing [<sup>3</sup>H]-leucine. To quantitate total newly synthesized proteins, medium and cells were harvested and precipitated with a final concentration of 10% trichloroacetic acid [1,10]. Immunoreactive collagenase was precipitated from the medium and cells as described earlier [1,10]. Two methods were used to assess the specificity of this process. First, <sup>3</sup>H-labeled material which was immunoprecipitated from the medium was solubilized by boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer and subjected to SDS-PAGE (see below). After drying, the gel was exposed for fluorography. Second, as a measure of the specificity of precipitation, replicate samples of culture medium or cell lysate were precipitated with antiserum to collagenase or with ovalbumin and antiovalbumin serum as a control precipitating system [1]. Specific collagenase was taken as that amount of radioactivity precipitated by specific anticollagenase antiserum minus that precipitated in the ovalbumin-antiovalbumin system [1].

#### Other Assays

Analytical SDS-PAGE of the washed immunoprecipitates was carried out as follows. The washed immunoprecipitates were dissolved in 25–100 µl of sample buffer containing 0.065 M Tris-HCl (pH 6.8), 8 M urea, 3% SDS and 2% β-mercaptoethanol for gel electrophoresis. The precipitates were dissolved in a boiling water bath for 5 min. The samples were applied to a discontinuous SDS slab gel (1 mm thick) made according to King and Laemmli [28] with 10% acrylamide and 0.27% N,N'-methylenebisacrylamide in the separating gel. Electrophoresis was carried out at 70 mA/mm slab thickness. The different gel slabs were either stained with Coomassie Brilliant Blue or processed for fluorography. For fluorography, the slab gels were equilibrated with dimethylsulfoxide, immersed in 20% 2,5-diphenyloxazole in dimethylsulfoxide for 3 hr, rinsed in distilled water for 20 hr, and dried under vacuum. The dried gels were exposed to Kodak XR-5 X-Omat R film. Protein was measured by the method of Lowry et al [29]. DNA was determined in the cell layer [30].

### RESULTS

We first examined the effect of adding varying concentrations of colchicine to the serum-free culture medium. As shown in Fig 1, the addition of 10<sup>-9</sup> to 10<sup>-6</sup> M concentrations of colchicine produced a progressive stimulation of collagenase activity released into the culture medium, so that at 10<sup>-6</sup> M there was an approximate 3-fold stimulation in collagenase expression. The specificity of this effect was assessed by determining the response of the cells to colchicine as compared to their response to the biologically inert photoisomer, lumicolchicine (Table I). In this case, under conditions that gave about a 3-fold increase in collagenase activity, lumicolchicine produced a negligible

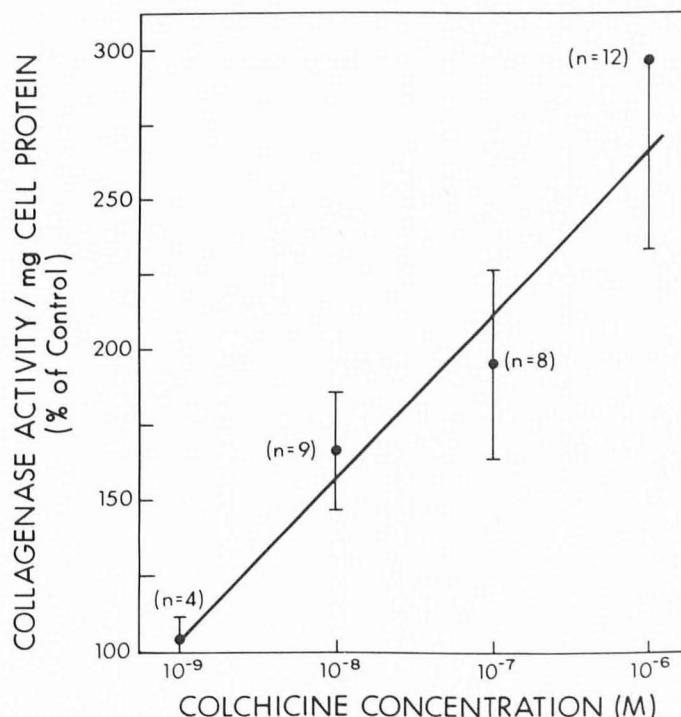


FIG 1. Effect of varying colchicine concentrations on collagenase expression in normal human skin fibroblast cultures. Confluent or near-confluent normal skin fibroblast cultures were maintained for 24 hr in serum-free culture medium containing the indicated concentrations of colchicine. Collagenase activity was determined as total cpm [<sup>14</sup>C]-collagen solubilized per mg cell protein in a 5-hr assay at 37°C. The data were pooled from 3 different experiments and are expressed as a % of the activity found in control cultures containing no colchicine. The numbers in parentheses represent the number of cultures examined at each concentration.

TABLE I. Specificity of the colchicine effect on collagenase expression in normal fibroblast cultures<sup>a</sup>

Culture	Colchicine concentration (M)	Collagenase activity <sup>b</sup>	
		(cpm × 10 <sup>-3</sup> / mg)	(% of control)
Control (n = 3)	0	11.7 ± 1.6	100
Lumicolchicine (n = 2)	10 <sup>-6</sup>	12.7 ± 5.4	109
Colchicine (n = 4)	10 <sup>-6</sup>	34.4 ± 30.2	294

<sup>a</sup> Confluent 75-cm<sup>2</sup> culture flasks were placed in serum-free medium for 24 hr. The numbers in parentheses refer to the number of cultures examined.

<sup>b</sup> Collagenase activity is expressed as total cpm [<sup>14</sup>C]-collagen solubilized per mg cell protein (mean ± SD) in a 5-hr assay at 37°C.

response in terms of collagenase activity found in the culture medium.

As noted for Fig 1, substantial variability was observed in the response of the various cultures to any given concentration of colchicine. This was true not only among various cell strains but also was observed within the same cell strain in different experiments. These findings suggested that each cell strain might manifest a differential susceptibility to colchicine stimulation. Since our previous studies had shown that culture density itself affected collagenase synthesis [21], we reasoned that the effect of colchicine might be modulated by the stage of culture growth. As depicted in Fig 2, despite the fact that these cultures were exposed to colchicine only under conditions independent of cell growth (i.e., in serum-free medium), their response to this drug was found to be dependent on the degree of confluence of the monolayer. Cultures that were in a subconfluent state of growth prior to being placed in serum-free, colchicine-containing medium were the most responsive (e.g.,

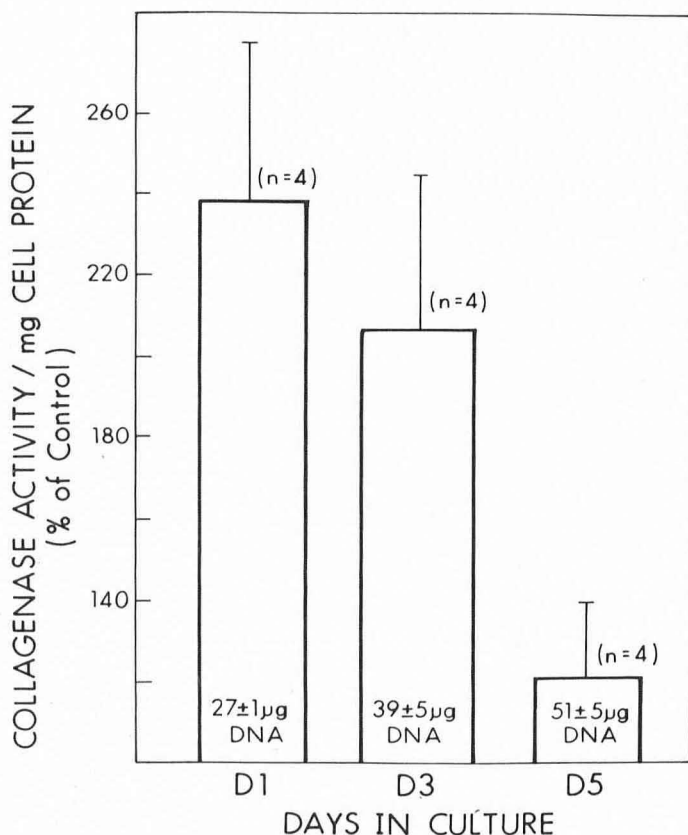


FIG 2. Effect of culture density on the response of normal human skin fibroblasts to colchicine. Normal skin fibroblasts were grown in the presence of fetal calf serum. On days 1 (D1), 3 (D3), and 5 (D5), 4 replicate cultures were placed in serum-free medium containing  $10^{-6}$  M colchicine for 24 hr. At the end of this serum-free incubation, medium was harvested for determination of collagenase activity as described in the legend to Fig. 1. DNA was determined in the cell layer.

D1 and D3). In contrast, cells that had reached full confluence were much less responsive in displaying increased expression of collagenase (e.g., D5).

We next examined the possible mechanisms for the colchicine-induced stimulation of collagenase expression. Since human skin procollagenase exists in the culture medium in latent form and requires proteolytic activation [23,26], for each experiment, we performed a trypsin-activation titration. As shown in one such titration (Fig 3), in the absence of colchicine in the culture medium, maximum activity was observed with 0.2 µg of trypsin, whereas in the colchicine-containing culture medium, maximum activity was seen with 0.5 µg of trypsin. These findings, coupled with the fact that there was no stimulation of activity in the complete absence of trypsin, suggest that the medium in this case contained 2–2.5 times as much latent enzyme protein requiring activation as the control culture medium. Furthermore, the effect of colchicine on activity was specifically dependent upon including colchicine in the culture medium with the cells; direct addition of the drug to the collagenase assay produced no stimulation of catalysis.

In a second series of experiments, the effect of colchicine on both collagenase activity and immunoreactive protein was examined. As shown in Table II, in each case the colchicine-induced stimulation of collagenase activity was directly paralleled by an increase in immunoreactive enzyme protein. This resulted in no change in the catalytic efficiency (activity per unit immunoreactive protein) of the enzyme. Thus it seemed unlikely that the colchicine was in any way causing the cells to synthesize a structurally different collagenase. Furthermore, these results strongly suggested that the mechanism for colchicine action was increased synthesis of enzyme protein.

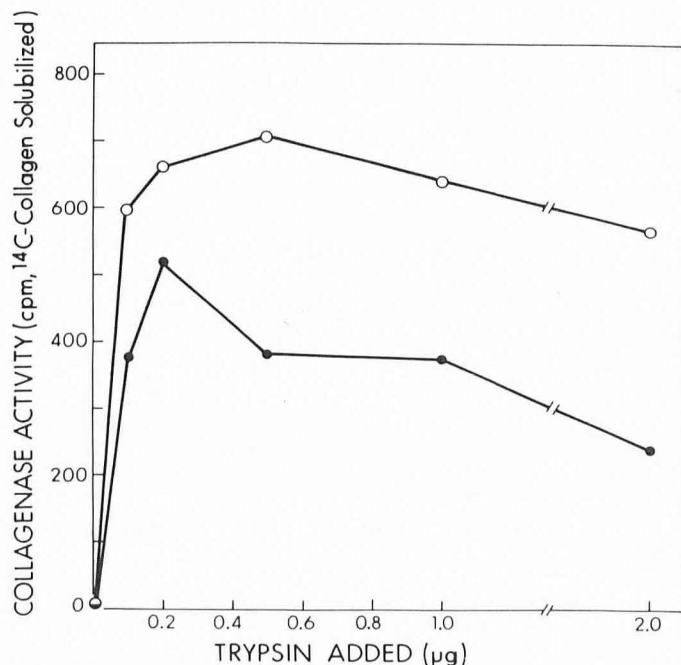


FIG 3. Proteolytic activation by human skin procollagenase. Crude culture medium (100 µl) containing latent procollagenase was activated by preincubation with varying concentrations of trypsin for 10 min at 25°C. Tryptic activity was then stopped by the addition of a 5-fold molar excess soybean trypsin inhibitor and the entire mixture was assayed for 1 hr at 37°C using [ $^{14}$ C]-collagen fibrils. Each point represents the mean of two assays. ●—●, Control cultures (no colchicine); ○—○, cultures containing  $10^{-6}$  M colchicine.

TABLE II. Effect of colchicine on the catalytic efficiency of collagenase<sup>a</sup>

Culture	Colchicine concentration (M)	Collagenase activity <sup>b</sup> (cpm × 10 <sup>-3</sup> /mg)	Immunoreactive protein <sup>c</sup> (ng × 10 <sup>-3</sup> /mg)	Catalytic efficiency <sup>d</sup> (cpm/ng)
<i>Exp 1</i>				
Control	0	1.1	0.1	11.0
Colchicine	$10^{-6}$	2.4	0.2	12.0
<i>Exp 2</i>				
Control	0	2.0	0.5	4.0
Colchicine	$10^{-6}$	4.6	0.8	5.8
<i>Exp 3</i>				
Control	0	8.6	2.7	3.2
Colchicine	$10^{-6}$	15.3	4.5	3.4
<i>Exp 4</i>				
Control	0	6.5	1.5	4.3
Colchicine	$10^{-7}$	8.8	2.0	4.4

<sup>a</sup> Confluent 75-cm<sup>2</sup> culture flasks of normal fibroblasts were placed in serum-free medium for 24 hr.

<sup>b</sup> Collagenase activity is expressed as total cpm [ $^{14}$ C]-collagen solubilized per mg cell protein during a 5-hr assay at 37°C.

<sup>c</sup> Immunoreactive collagenase was quantitated by radioimmunoassay and is expressed at total ng per culture per mg cell protein.

<sup>d</sup> Catalytic efficiency, activity per unit immunoreactive protein.

To explore this possibility further, we examined the effect of colchicine on the biosynthesis of procollagenase. Fig 4 shows a fluorograph of newly synthesized  $^3$ H-labeled procollagenase immunoprecipitated from colchicine-containing culture medium using specific antiserum to collagenase. Two species of procollagenase having molecular weights of approximately 60,000 and 55,000 were synthesized, a finding identical to that seen previously under normal biosynthetic conditions [1]. In the control precipitate containing ovalbumin and antiovalbumin, no distinct labeled proteins could be seen, indicating the high degree of specificity of this method for assaying the synthesis of collagenase.



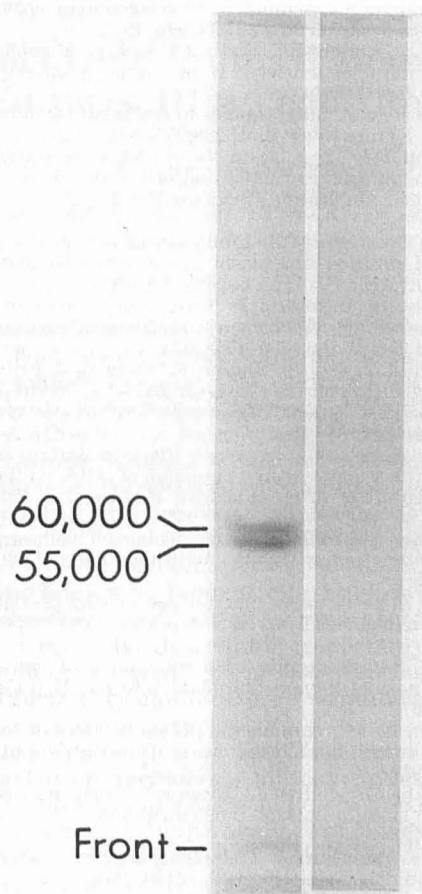


FIG 4. Fluorographs of SDS-PAGE patterns after immunoprecipitation from fibroblast culture medium. The *left* gel depicts the pattern of immunoreactive collagenase protein precipitated from colchicine-containing medium using antiserum to human skin collagenase. The *right* gel shows the pattern obtained with the ovalbumin-antiovalbumin control precipitating system. The 60,000 and 55,000 positions mark the migration of authentic purified human skin procollagenase forms.

Using this system, the kinetics of the effect of colchicine on collagenase expression were characterized (Fig 5). Compared to the rate of collagenase synthesis in the absence of the drug (Fig 5A), the addition of  $10^{-6}$  M colchicine to the culture medium resulted in an approximate 2-fold increase in the rate of synthesis (Fig 5B). This effect could be seen within 1.5 hr of exposure to colchicine as manifested by a marked increase in the slope of the curve of secretion. Our previous studies have shown that once the intracellular  $^3\text{H}$ -labeled collagenase pool reaches a constant level, the rate of secretion is reflective of de novo synthesis [1]. This increase in enzyme synthesis occurred without a demonstrable change in the rate of total protein synthesis under the same conditions. In this case, total newly synthesized proteins (i.e., in the medium + cells) were the same in the absence (Fig 5C) and presence (Fig 5D) of colchicine, suggesting that the effect of colchicine on collagenase may be specific.

#### DISCUSSION

In the present study we have shown that the microtubule-disruptive agent, colchicine, enhanced—in a dose-dependent fashion—the expression of collagenase in cultures of normal human skin fibroblasts. Such a finding could in theory be attributed to increased enzyme synthesis, to an altered catalytic state of the enzyme, or to diminished degradation of the enzyme, as originally suggested by Harris and Krane [19] in the synovial explant system. However, investigations both of the catalytic efficiency of the enzyme (Table II) and of the biosyn-

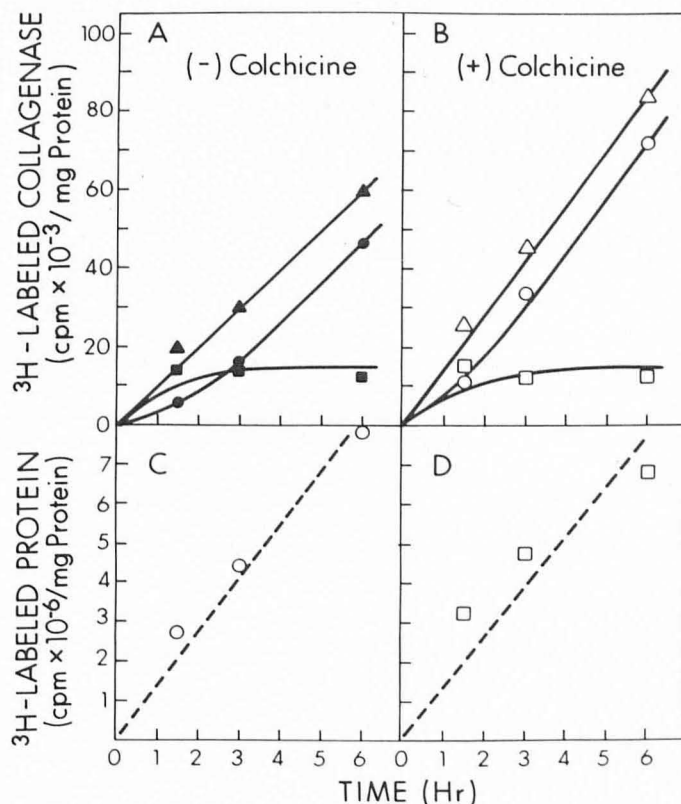


FIG 5. Effect of  $10^{-6}$  M colchicine on the biosynthesis of collagenase and total protein in normal human skin fibroblast cultures. Replicate  $10\text{-cm}^2$  culture plates containing near-confluent skin fibroblast cultures were placed in serum-free medium containing 0 or  $10^{-6}$  M colchicine and approximately  $10 \mu\text{Ci}/\text{ml}$  of  $^3\text{H}$ -leucine. Medium and cells were harvested at the indicated times for determination of immunoreactive collagenase and total 10% trichloroacetic acid-precipitable protein. A and C, No added colchicine.  $\blacksquare$ — $\blacksquare$ , Intracellular collagenase;  $\bullet$ — $\bullet$ , extracellular collagenase;  $\blacktriangle$ — $\blacktriangle$ , total collagenase synthesis;  $\circ$ — $\circ$ , total protein synthesis. B and D,  $10^{-6}$  M colchicine.  $\square$ — $\square$ , Intracellular collagenase;  $\circ$ — $\circ$ , extracellular collagenase;  $\triangle$ — $\triangle$ , total collagenase synthesis;  $\square$ — $\square$ , total protein synthesis.

thesis of the enzyme (Fig 5) suggest that the primary mechanism involved was stimulation of collagenase synthesis. Furthermore, compared to the striking failure to affect general protein synthesis (Fig 5), colchicine displayed a noteworthy degree of specificity on collagenase expression. Indeed, in biosynthetic experiments (Fig 5), after a 6-hr exposure to  $10^{-6}$  M colchicine, total collagenase synthesis (medium + cells) represented approximately 0.75% of the newly synthesized proteins in the absence of colchicine but accounted for 1.25% of total proteins synthesized in the presence of this drug.

Although indirect, the evidence suggests that the colchicine-induced modulation of collagenase synthesis was related to its effects on microtubules. In this regard, only colchicine—not its photoisomer, lumicolchicine—produced a stimulatory effect on collagenase (Table I), a finding that correlates with the relative capacities of these two drugs to bind to tubulin [31]. Indeed, the capacity of colchicine to bind specifically to tubulin has resulted in its widespread use as a diagnostic tool for the participation of microtubules in various cellular processes [32].

One intriguing, but as yet not fully explained, aspect of these studies was the observation that subconfluent fibroblast cultures were most responsive to the effects of colchicine (Fig 2). It is essential to emphasize that these experiments were performed under conditions independent of cell division; i.e., the cultures were maintained only in serum-free medium, a condition that does not support cell multiplication. In addition, cells arrested in metaphase were not seen. Thus it is unlikely that the colchicine-induced modulation of collagenase could be at-

tributed to an effect of the drug on cell division. Nevertheless, these studies do not exclude the possibility that the drug might have, in a sense, "trapped" fibroblasts in a phase of the cell cycle during which collagenase synthesis was enhanced. Our current methods do not permit us to address this possibility, and it is likely that the question will be answered only by cell synchronization experiments.

Biologically these studies imply that microtubules in general, or possibly several populations of microtubules, behave differently depending upon the cell product in question. For example, the secretion of certain structural macromolecules, such as collagen [13-16] and proteoglycans [17], is blocked by colchicine. In contrast, similar mesenchymal tissues [19] and cells [20] respond to identical concentrations of colchicine by increasing the synthesis of collagenase. Such a paradox is not easily resolved without postulating the existence (a) of two or more populations of cells, (b) of multiple populations (or types) of microtubules, or (c) of a functional modulation possibly involving microtubule-associated proteins [32]. Irrespective of whether any of these is the case, the capacity of a microtubule-active drug to stimulate collagenase synthesis could serve as a useful probe to the role of microtubules in diseases involving this enzyme.

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